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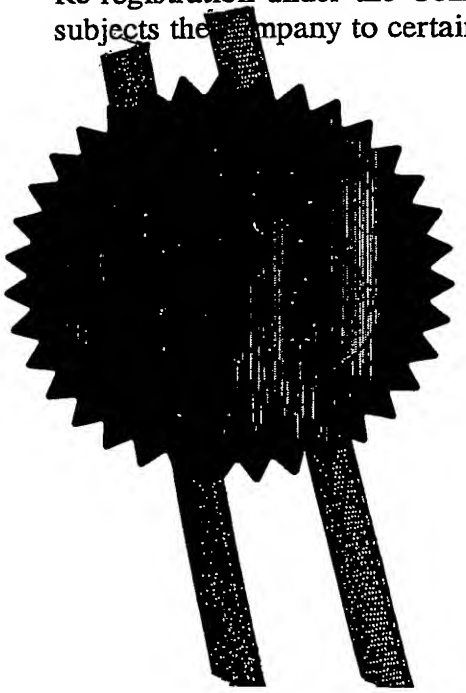

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2.	Patent application number *(The Patent Office will fill in this part)*	02 OCT 2003 0323089.3
3.	Full name, address and postcode of the or of each applicant *(underline all surnames)* 07143563001 Patent ADP number *(if you know it)* If the applicant is a corporate body, give the country/state of its incorporation	NOVARTIS NUTRITION AG MONBIJOUSTRASSE 118 3001 BERN SWITZERLAND SWITZERLAND
4.	Title of invention	Organic Compounds
5.	Name of your agent *(If you have one)* "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)* Patents ADP number *(if you know it)*	Craig McLean Novartis Pharmaceuticals UK Limited Patents and Trademarks Wimblehurst Road Horsham, West Sussex RH12 5AB 07181522002 ✓

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Craig McLean

12. Name and daytime telephone number of person to contact in the United Kingdom: Mr. Trevor Drew 01403 323069

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Organic Compounds

The present invention concerns a method for providing a nutritional or pharmaceutical composition comprising a prebiotic to an individual in need thereof.

Dietary interventions for the treatment or prevention of diseases are highly sought after to relieve the burdens on medical service providers. One of the most promising current targets for such dietary interventions is the gastrointestinal tract. The large intestine is, by far, the most densely populated area of the gut. Its resident microflora appears to be associated with control of transit time, bowel habit, absorptive and mucosal function as well as implicated, through its normal metabolic state, in many other physiological processes relevant to host health and disease. Modulation of the composition of this microflora through use of certain food ingredients, offers an attractive basis for developing novel dietary therapies. This has resulted in the development of pro and prebiotic concepts. Both rely upon enhancement of beneficial components of the microflora, such as bifidobacteria and lactobacilli. Whilst the former concept uses a live microbial supplement, the latter is defined as a nondigestible food ingredient which selectively stimulates growth and/or activity of these beneficial groups of bacteria.

Health claims attributed to the consumption of probiotics have been reported for a long time. However, one of the main problems of this approach is survival of the probiotic in the complex gut ecosystem. Following ingestion, strains are confronted by factors such as gastric acids, bile salts and various enzymes as well as the ability to establish in the large intestine and compete with a diverse and metabolically active indigenous microbiota. An alternative to the survivability problems lies in the use of prebiotics and it may be assumed that these will be used as predominant ingredients in future developments of nutritional approaches for the treatment or prevention of diseases and disorders concerning the gastrointestinal tract.

Prebiotics are non-digestible food ingredients which have a beneficial effect on the health. For a food ingredient to be classified as a prebiotic it must fulfill the following criteria: i) neither be hydrolyzed nor absorbed in the gastrointestinal tract, ii) be selectively fermented

by one or a limited number of potentially beneficial bacteria commensal to the colon, such as lactobacilli and bifidobacteria, which are stimulated to grow and/or become metabolically activated, iii) be able to alter the colonic microflora towards a healthier composition, by increasing, for example, numbers of saccharolytic species while reducing putrefactive microorganisms. The fermentation of the prebiotic by the colonic bacteria may lead to the production of short chain fatty acid (SCFA), such as succinic, lactic, formic, acetic, propionic, isobutyric, butyric, isovaleric and valeric acids, hydrogen and carbon dioxide gases.

In recent years, there is on the part of the consumers an increasing demand for foodstuffs that in addition to having a nutritional value also have a positive impact on health. In particular there is an interest in developing functional foods having prebiotic capability. *In vivo* human studies have shown that dietary addition of particular oligosaccharides, such as fructooligosaccharide (FOS), may lead to an increase in beenficial faecal bifidobacteria. Nevertheless, there is no uniform method to determine the effectiveness of different fibers to act as prebiotics and to compare their prebiotic capabilities.

There is a need to develop a method that will allow quantification and/or comparison of the prebiotic capabilities of different fibers, in particular to provide improved nutritional compositions containing prebiotics.

In one aspect of the invention there is provided a comparative and standardized method for assessing the prebiotic capability of a dietary fiber, e.g. an oligosaccharide, and/or a comparative and standardized method for quantifying the effectiveness of a prebiotic, in particular through its effect on faecal bacteria. Preferably the method is a subtractive culture method.

As used herein, a "subtractive culture method" refers to a method which may include at least the following steps: 1- incubating a faecal bacterial culture in parallel in the presence and in the absence of the tested fiber during a certain incubation period, e.g. until the substrate or the tested fiber is completely fermented; 2- determining the amount of the faecal bacteria in the culture in the presence and in the absence of the tested fiber, in particular the amount of beneficial or potentially beneficial faecal bacteria in the presence and in the absence of the tested fiber and the amount of non beneficial faecal bacteria in the same conditions; and 3- comparing the amount of faecal bacteria in the culture in the presence

and in the absence of the tested fiber, in particular the amount of beneficial or potentially beneficial faecal bacteria versus non beneficial faecal bacteria.

The subtractive culture method of the invention may allow the evaluation or quantification of the effect of the tested fiber on the growth of faecal bacteria, in particular of beneficial or potentially beneficial faecal bacteria. Such an evaluation or quantification may be made by subtracting the amount of the faecal bacteria, in particular beneficial or potentially beneficial faecal bacteria, in the culture in the absence of the tested fiber from the amount of the faecal bacteria, in particular beneficial or potentially beneficial faecal bacteria, in the culture in the presence of the tested fiber. The value obtained with such a calculation may be reflective of the prebiotic capability of the tested fiber.

In another embodiment of the invention, the subtractive culture method of the invention may allow the evaluation or quantification of the effect of the tested fiber on the modification of the faecal bacteria population, in particular towards a healthier composition. In such an evaluation or quantification method, the step 3) as hereinabove described may include the following sub-steps: 3.1) subtracting the amount of the beneficial or potentially beneficial faecal bacteria in the culture in the absence of the tested fiber from the amount of the beneficial or potentially beneficial faecal bacteria in the culture in the presence of the tested fiber; 3.2) subtracting the amount of the non beneficial faecal bacteria in the culture in the absence of the tested fiber from the amount of the non beneficial faecal bacteria in the culture in the presence of the tested fiber, and 3.3) subtracting the value as determined in step 3.2) from the value as determined in step 3.1) to get a value which may be reflective of the prebiotic capability of the tested fiber.

In another aspect of the invention there is provided a method for designing a prebiotic containing composition effective in controlling, e.g. treating, preventing or ameliorating diseases of the gastrointestinal tract such as chronic gut disorder, irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, colon cancer and associated disorders.

In another aspect of the invention there is provided a method for designing a prebiotic containing composition effective in stimulating the growth of endogenous bifidobacteria

and/or lactobacilli, and/or inhibiting the growth of Bacteroides, Eubacteria, Clostridia, Coliforms and/or Sulfate reducing Bacteria.

In yet another aspect of the invention there is provided a method for designing a nutritional or pharmaceutical composition comprising prebiotics, which method comprises
(a) evaluation, e.g. quantification of the prebiotic capability of a fiber, and/or identification of a prebiotic, by a subtractive culture method, and
(b) formulation of a nutritional or pharmaceutical composition comprising the fiber, e.g. the prebiotic, evaluated and/or identified in step (a) and a nutritionally or pharmaceutically acceptable carrier.

As used herein, the "prebiotic capability of a fiber " refers to the capability of a fiber to act as a prebiotic, e.g. to be fermented by beneficial or potentially beneficial faecal bacteria, such as lactobacilli and bifidobacteria, and/or to be able to alter the faecal bacterial population towards a healthier composition, e.g. in stimulating the growth and/or the metabolism of beneficial or potentially beneficial faecal bacteria and/or in inhibiting the growth and/or the metabolism of non beneficial or pathogenic faecal bacteria. Since faecal bacteria are bacteria originating in the long intestine, the method of the invention may allow determination of prebiotic effect in vivo.

According to the invention there is provided a method for designing a nutritional or pharmaceutical composition comprising at least one prebiotic and delivering said nutritional or pharmaceutical composition to an individual in need thereof, which method comprises, e.g. consists of,
(a) evaluation, e.g. quantification of, the prebiotic capability of a fiber, and/or identification of a prebiotic,
(b) formulation of a nutritional or pharmaceutical composition comprising the prebiotic, e.g. the fiber, evaluated and/or identified in step (a) and a nutritionally or pharmaceutically acceptable carrier, and
(c) providing the nutritional or pharmaceutical composition obtained in step (b) to an individual in need thereof.

According to the invention, the step (a) may comprise the step of, quantifying the effect of the tested fiber, or blend of tested fiber, on faecal bacteria, e.g. on faecal bacterial growth

and/or on changes in faecal bacterial population, e.g. on the multiplication and/or the metabolism of the faecal bacteria. Accordingly, step (a) may comprise the step of monitoring and/or quantifying the effect of the tested fiber, or blend of tested fiber, in stimulating the multiplication and/or activating the metabolism of beneficial or potentially beneficial faecal bacteria, such as e.g. lactobacilli and/or bifidobacteria, and/or in inhibiting the multiplication and/or the metabolism of non beneficial and/or detrimental faecal bacteria, such as e.g. saccharolytic and/or putrefactive species.

In one embodiment of the invention, the step (a) may be carried out in monitoring the growth of faecal bacterial populations, e.g. in anaerobic batch cultures containing the tested fiber, or blend of tested fibers, e.g. as sole carbon source, and inoculated by faecal bacteria, for instance in the form of faecal samples. The faecal samples may be obtained from a healthy human and/or a human who did not receive any antibiotic treatment for at least 6 months. Sucrose may be used as a control substrate as it is not selective, i.e. may be fermented by all bacteria. As a negative control culture medium without any substrate, e.g. without any fiber or any sugar, may be used.

In one aspect of the invention, step (a) may comprise a comparative and standardised method, e.g. a calculation method.

In one aspect of the invention, step (a) may comprise the step of comparing the effect of the tested fiber, or blend of tested fibers, on faecal bacteria to the effect of another fiber, e.g. a known prebiotic, to the same faecal bacteria in the same conditions.

In another aspect of the invention, step (a) may comprise a process for determining the "prebiotic index" (PI) of a fiber. The basic concept of PI is to derive a single number that quantifies the effect of the fiber on the bacterial, e.g. the faecal bacterial population, e.g. on the amount of beneficial faecal bacteria versus non beneficial and/or pathogenic faecal bacteria.

The following equation may be used to describe the prebiotic index:

$PI = \Delta B + \Delta L + \Delta E - \Delta Ba - \Delta Cl - \Delta Co - \Delta SRB$

wherein PI = prebiotic index; Δ = amount of bacteria, e.g. log_{10} bacterial populations, in presence of the fiber minus the amount of bacteria, e.g. log_{10} bacterial populations, in absence of the fiber; B = bifidobacteria, e.g. *Bifidobacterium* spp; L = lactobacilli, e.g. *Lactobacillus* spp; E = Eubacteria, e.g. *Eubacterium* rectale; Ba = bacteroides, e.g. *Bacteroides* spp ; Cl = clostridia, e.g. *Clostridium* coccoides, *Clostridium* histolyticum; Co = coliforms, e.g. *Escherichia* coli and SRB = Sulfate Reducing bacteria, e.g. *Desulfovibrio* spp.

The prebiotic index may be calculated by i) summing increases of amounts, e.g. log_{10} populations, of beneficial and potentially beneficial faecal bacteria, e.g. bifidobacteria, lactobacilli and/or Eubacteria and ii) adding the decrease or subtracting the increase to the numerical value obtained for the amounts, e.g. log_{10} populations, of non beneficial faecal bacteria, e.g. clostridia, bacteroides, coliforms and/or Sulfate Reducing bacteria, as determined e.g. in anaerobic fermentation culture, e.g. in a batch culture and/or a fermentation vessel.

In one embodiment of the invention, a fiber which gives a Prebiotic Index as hereinabove described, greater than 0, preferably greater than 0.5 or greater than 1 may be considered as a good and suitable prebiotic.

According to the invention, step (a) may comprise the step of quantifying the capacity of the tested fiber, or blend of tested fiber, to be fermented by the faecal bacteria, e.g. by the beneficial and potentially beneficial faecal bacteria. The capacity of the tested fiber to be fermented may be monitoring or quantifying e.g. through the production of fermentation end product, such as short chain fatty acids (SCFA), the rate of the fiber breakdown, and/or the fermentation time, e.g. the time necessary for the fiber to be fermented, e.g. completely fermented, e.g. by the beneficial and potentially beneficial faecal bacteria.

In one aspect of the invention, step (a) according to the invention may comprise a process for determining the "measure of the prebiotic effect" (MPE). The basic concept of MPE is to derive a single number that quantifies the prebiotic capability of the fiber, e.g. its effect on the faecal bacterial, e.g. on the faecal bacterial growth and/or faecal bacterial population changes, e.g. as determined by the PI, and/or represents its capacity to be fermented by the faecal bacteria, e.g. to induce the production of fermentation end product, such as short chain fatty acids (SCFA). The prebiotic capability of the fiber, e.g. the MPE, may be

evaluated, e.g. quantified, by monitoring the production of fermentation end products, such as short chain fatty acids (SCFA). The prebiotic capability of the fiber, e.g. the MPE, may also be evaluated, e.g. quantified, by analyzing, e.g. quantifying the rate of the fiber breakdown. The prebiotic capability of the fiber, e.g. the MPE, may further be evaluated, e.g. quantified, by analyzing the fermentation time of the tested fiber, and/or the relationship between the faecal bacterial population growth and the substrate, e.g. fiber, concentration.

In another aspect of the invention there is provided a method for designing a nutritional or pharmaceutical composition comprising at least one fiber with a prebiotic capability, e.g. at least one prebiotic, and delivering said nutritional composition to an individual in need thereof, which method comprises

(a) determination of the measure of the prebiotic effect (MPE) and optionally comparing the tested fiber to another fiber, e.g. to a prebiotic,

(b) formulation of a nutritional or pharmaceutical composition comprising the fiber, e.g. the prebiotic, identified in step (a) and a nutritionally or pharmaceutically acceptable carrier, and

(c) providing the nutritional or pharmaceutical composition obtained in step (b) to an individual in need thereof.

According to the invention the MPE may include, e.g. may be defined by, the prebiotic index, as hereinabove described.

In another aspect of the invention, the MPE may include, e.g. may be defined by, the fermentation pattern of the tested fiber, e.g. the measure of the fermentation end products, e.g. short chain fatty acids (SCFA). The MPE may further include, e.g. may be defined by, the relationship between the production of fermentation end products, e.g. SCFA, and the concentration of substrate, e.g. fiber.

In a further aspect of the invention, the MPE may include, e.g. may be defined by, the measure of the fiber breakdown, e.g. the rate of fiber breakdown.

In yet a further aspect of the invention, the MPE may include, e.g. may be defined by, the determination of the fermentation time, e.g. the time necessary for the fiber to be fermented,

e.g. completely fermented. The MPE may include, e.g. may be defined by the relationship between the faecal bacterial population growth and the substrate, e.g. fiber, concentration.

In one embodiment of the invention the MPE may be described by the following equation:

$$\frac{1}{2}\sqrt{x^2y^2 + x^2z^2 + y^2z^2} = MPE$$

wherein
x = changes in the bacterial population induced by the tested fiber, e.g. as measured by PI;
y= quantification of the fermentation end products, e.g. SCFA production, e.g. succinic, lactic, formic, acetic, propionic, isobutyric, butyric, isovaleric and valeric acids production, or relationship between fermentation end products, e.g. SCFA production, and substrate, e.g. fiber, concentration ;
z= rate of fiber breakdown, e.g. in relation to the bacterial growth, or relationship between substrate, e.g. fiber, concentration and bacterial growth.

As used herein, "faecal bacteria" refers to indigenous or commensal bacteria of the colon, including beneficial and potentially beneficial bacteria, such as Bifidobacteria, Lactobacilli, Eubacteria, and non beneficial, e.g. detrimental, putrefactive or pathogenic bacteria, such as bacteroides, Clostridia, Coliforms, Sulfate Reducing bacteria (SRB). Faecal bacteria may be single bacteria species or may be mixtures of different species. Faecal bacteria may derive from natural sources, such as faecal samples from human, e.g. healthy human and/or human who did not take any antibiotics for at least 6 months. Faecal bacteria may be or not purified.

For the purpose of the invention, the term " fiber" refers to fibers, e.g. dietary fibers, e.g. soluble or insoluble fibers, e.g. hydrolyzed fibers. In particular the fibers according to the invention are able to undergo fermentation in the colon to produce short chain fatty acids (SCFA), such as e.g. succinic, lactic, formic, acetic, propionic, isobutyric, butyric, isovaleric or valeric acids or hydrogen and carbon dioxide gases.

One example of fibers according to the invention are fructo-oligosaccharides (also called oligofructose) (FOS), oligosaccharides that are members of the inulin subclass of fructans. FOS occur in nature in many kind of plants, including onions, garlic, shallots, wheat, rye,

bananas, aspergus, tomatoes, artichokes, dahlia and chicory root. FOS can be produced enzymatically, through chemical techniques or by extraction from natural substances. Short chain FOS are composed of one to three fructose molecules linked to one molecule of sucrose: their polymerization degree (DP) is not higher than 6, and they can be synthesized from sucrose through the use of transfructosylating enzymes. Treatment of sucrose with these transfructosylating enzymes results in a mixture of FOS containing 2, 3 or 4 fructose units, such as 1-kestose, nystose and fructosyl-nystose. *In vivo* human studies have been shown that dietary addition of FOS leads to an increase in faecal bifidobacteria and is a very effective prebiotic.

As used herein the term "FOS" encompasses FOS and short chain FOS. FOS may comprise between 2 and 20 saccharide units, for example between 2 to 15 saccharide units, further example between 2 to 7 saccharide units or between 2 to 6 saccharide units. For example FOS may contain about 95% by weight disaccharides to heptasaccharides, based on the total weight of FOS.

Oligofructose is commercially available, for example as Actilite,RAFTILOSE@ from ORAFTI, (Tienen, Belgium), in various grades such as, for example, RAFTILOSE@ P95 which contains about 95 % by weight oligofructose, composed of chains with a degree of polymerisation ranging from 2 to about 7, typically with a (DP) of 3.5 to 4.5, and containing about 5 % by weight in total of glucose, fructose and sucrose.

Another example of fibers according to the invention are galacto-oligosaccharides (GOS) , which may comprise di, tri, tetra, penta and hexasaccharides, mainly consist of galactose as a sugar component, and are formed by the action of beta-galactosidase on lactose. GOS may comprise between 2 and 15 saccharide units, for example between 2 to 10 saccharide units, further example between 2 to 7 saccharide units or between 2 to 6 saccharide units. For example GOS may contain about 0 to about 45% of weight disaccharides, further example about 10 to about 40% of weight disaccharides, about 20 to about 35% of weight disaccharides, or about 33% of weight disaccharides, based of the total weight of GOS. For example GOS may contain about 0 to about 50% of weight trisaccharides, further example about 10 to about 45% of weight trisaccharides, about 20 to about 40% of weight trisaccharides, or about 39% of weight trisaccharides, based of the total weight of GOS. For example GOS may contain about 0 to about 50% of weight tetrasaccharides, further

example about 5 to about 45% of weight tetrasaccharides, about 10 to about 40% of weight tetrasaccharides, or about 18% of weight tetrasaccharides, based of the total weight of GOS. For example, GOS may contain about 0 to about 30% of weight pentasaccharides, further example about 1 to about 25% of weight pentasaccharides, about 2 to about 10% of weight pentasaccharides, or about 7% of weight pentasaccharides, based of the total weight of GOS.

GOS is commercially available, for example under the trade name Vivinal GOS or Elix'or GOS.

As used herein the term "GOS" encompasses GOS as hereinabove defined and trans Galacto-oligosaccharides, also called tGOS.

Hydrolysed fiber may be derived from numerous known fibers. Preferred hydrolysed fibers include hydrolysed guar gum, e.g. partially hydrolyzed guar gum. The term hydrolysed fibers as used herein refers to fibers hydrolysed in conventional manner, e.g. chemically or enzymatically to fibers having a reduced molecular weight, which hydrolysed products may be tube compatible when administered at the desired daily amount.

An example of hydrolysed guar gum is Benefiber®, e.g. as described in U.S. Patent No.5,260,279, which is hereby incorporated by reference. Prior to hydrolysis, the molecular weight of guar gum is approximately 200,000; after hydrolysis it is 20,000-30,000. The molecular weight range of the hydrolysed guar gum may vary, preferably may be between 24 and 30 kDa.

For the method of the present invention anaerobic culture fermentations may be used to assess and measure e.g. bacterial growth, changes in bacterial population, fermentation times, rate of substrate, e.g. fiber, breakdown, the production of fermentation end product, e.g. SCFA. Batch culture fermentations may also be used for the method of the invention to determine the minimum and maximum concentration of substrate, e.g. fiber, the prebiotic capability of combination of test substrates, e.g. combinations of test fibers, such as FOS: GOS, e.g. FOS: GOS (50:50), FOS: Benefiber®, e.g. FOS: Benefiber® (90:10) and GOS: Benefiber®, e.g. GOS: Benefiber® (90:10). Sucrose may be used as a control substrate as it

is not selective and will therefore be fermented by all bacteria. As a negative control culture medium without any substrate may be used.

The incubation of the faecal bacterial culture may be carried out in a volume comprised between about 100ml and 500ml, e.g. between about 100 ml and 400 ml, e.g. between 150 and 300ml, e.g. between 250 and 300ml. Preferably the incubation volume may be about 150 ml, or about 250ml, e.g. 270ml, or bout 300ml.

The incubation of the faecal bacterial culture may be carried out until the substrat e.g. the fiber, has been fermented, e.g. completely fermented. A period of fermentation may last for at least one day and up to about 15 days, e.g. between 1 day and 11 days, e.g. between 10 and 11 days. Preferably the incubation period may be of about 24 hours.

According to the invention, gut model experiments may be used e.g. to assess the persistence of test substrates, e.g. fibers, e.g. combination of fibers, through the colon; to assess the effect of each substrate, e.g. fiber, on bacterial growth and/or SCFA production. The gut model has been validated against gut contents from sudden death victims and gives a very close analogy to bacterial composition and activities in different areas of the large intestine.

For the method of the present invention bacterial population growth and changes may be determined by fluorescence *in situ* hybridisation (FISH), a culture independent molecular technique employing 16S rRNA oligonucleotide probes labelled with fluorescent dyes (See table1). The FISH method allows the visualization and localization of whole bacterial cells *in situ* in environmental samples. It will be appreciated that such a method is readily known to one skilled in the art.

For the method of the present invention, the production of SCFA may be determined by a technique readily known to one skilled in the art, such as HPLC or phenol-sulphuric acid assay.

In one aspect of the invention, there is provided a method for providing a nutritional or pharmaceutical composition containing a suitable prebiotic, e.g. a fiber having a prebiotic

Index as hereinabove described superior than 0, e.g. positive, preferably superior that 0.5, even more preferably superior than 1.

According to the invention, nutritional compositions refer to nutritional formulations, typically nutraceuticals, dietary supplements, functional food, beverage products, or food additives. Such nutritional compositions may be nutritionally complete, i.e. may include vitamins, minerals, trace elements as well as nitrogen, carbohydrate and fatty acid sources so that they may be used as the sole source of nutrition supplying essentially all the required daily amounts of vitamins, minerals, carbohydrates, fatty acids, proteins and the like. The nutritional compositions may also be in the form of low calorie formulations, e.g. low calorie meal replacements.

The compositions which can be designed by the method of the invention may be suited for oral or tube feeding.

Suitable product formats for the nutritional compositions include solutions, ready-for-consumption compositions, e.g. ready-to-drink compositions, instant drinks, liquid comestibles, like soft drinks, juices, sports drinks, milk drinks, milk-shakes, yogurt drinks or soups. Such compositions may also be designed in accordance with the method of the present invention in the form of a concentrate, a powder, or granules, e.g. effervescent granules, to be diluted in water or other liquid, such as milk or fruit juice.

Pharmaceutical compositions may be provided in the form of soft gels, sachets, powder, syrups, liquid suspensions, emulsions, solution, hard gelatin capsules or soft, sealed capsules consisting of gelatin and a plasticizer, such as glycerol or sorbitol.

The amount of fiber, e.g. prebiotics, contained in the compositions which are designed by the method of the invention may be determined in the light of various relevant factors including the purpose of administration, the age, sex and body weight of individual subject and the severity of the subject's symptoms.

The compositions which are designed by the method of the invention may also comprise any bioactive compounds or extracts which are known to have health benefits, especially compounds which have a beneficial influence on the gastro-intestinal tract, such as

probiotics, glutamine/glutamate or precursors thereof, or that inhibit bacterial adhesion to epithelial wall of the gastrointestinal tract, including mannans, galacturonic acid oligomers, preferably of natural origin.

The invention may be further illustrated by the following Examples:

Example

Unless otherwise stated, all chemicals and reagents are obtained from Sigma - Aldrich Co. Ltd. (Poole-UK) or BDH Chemicals Ltd. (Poole, UK). Testing substrates are listed in Table 2. Where required, culture pH is adjusted with either 1M HCl or 1M NaOH. Sterilisation is achieved by autoclaving at 121°C for 15 min.

(1) Preparation and collection of faecal samples

Faecal samples are obtained from healthy human volunteers. Volunteers are required not to have been prescribed antibiotics for at least 6 months prior to the study and have no history of gastrointestinal diseases. The samples are collected on site and used immediately following collection. A 1/10 dilution in anaerobic phosphate buffer (0.1M, pH 7.4) is prepared and the samples homogenised in a stomacher for 2 minutes.

Table 2. Test substrates and their composition

Type	Commercial name	Composition	Manufacturer
FOS	Actilight 950P®	95% oligosaccharides	Eridania Beighin Meiji, Neuilli sur Seine, France
tGOS	Elix'or®	58% tGOS, 19% glucose, 19% lactose, 0.8% galactose, 3% moisture	Borculo Domo, Netherlands
Partially hydrolysed guar gum	Benefiber®	80% soluble fibre, 12.5% carbohydrate, 5% moisture,	Taiyo Kaguku Co., Yokkaichi, Japans

(PHGG)		1.5% ash, 1% protein	
Guar gum	Guar gum		Sigma-Aldrich Co. Ltd., Poole,UK

(2) Batch Fermentations

Sterile, stirred, batch culture fermentation vessels (300ml volume) are filled with 135ml basal nutrient medium (peptone water 2g/l, yeast extract 2g/l, NaCl 0.1g/l, K_2HPO_4 0.04g/l, KH_2PO_4 0.04g/l, $MgSO_4.7H_2O$ 0.01g/l, $CaCl_2.6H_2O$ 0.01, $NaHCO_3$ 2g/l, Tween 80 2ml, Hemin 0.02g/l, Vitamin K_1 10µl, Cysteine.HCl 0.5g/l, Bile salts (sodium glycocholate and sodium taurocholate) 0.5g/l, pH7.0) and gassed overnight with oxygen free nitrogen. Prior to addition of the faecal slurry,0.25%, 0.5% or 1% (w/v) testing substrates are added to different vessels, culture temperature is set at 37°C by means of a circulating water bath and medium pH is maintained at 6.8 using an Electrolab pH controller. The vessels are inoculated with 15ml of fresh faecal slurry (1/10w/v) and continuously sparged with O_2-free N_2 at a flow rate of 15ml/min. Samples (3ml) from each vessel are obtained for fluorescence *in situ* hybridisation (FISH), analysis of SCFA by high performance liquid chromatography (HPLC) and total carbohydrate measurement by assay. Batch cultures are run over a period of 24 hours.

2.1. Fluorescence *in situ* hybridisation (FISH)

Differences in bacterial populations are assessed through use of FISH with oligonucleotide probes designed to target diagnostic regions of 16S rRNA. These are commercially synthesised and labelled with the fluorescent dye Cy3 (provided by Eurogentec UK Ltd). The molecular probes utilised are presented in Table 1. For total bacterial counts the nucleic acid stain 4,6-diamidino-2-phenylindole (DAPI) is used. Samples obtained from fermentation vessels are diluted in 4% (w/v) paraformaldehyde and fixed overnight at 4°C. The cells are then centrifuged at 1500 x g for 5 minutes, washed twice with phosphate-buffered saline (PBS; 0.1M, pH 7.0), resuspended in a mixture of PBS / 99% ethanol (1:1 w/v) and stored at –20°C for at least 1 hour. For Lab 158 probe samples are further resuspended in the enzyme mixture containing agents that increase cell permeability and incubated for 1 hour at 37°C. The cells are then washed in PBS, resuspended in 100% methanol and stored at –

20°C for at least 1 hour. The cell suspension is then added to the hybridisation mixture and left overnight to hybridise at the appropriate temperature for each probe. The hybridised mixture is vacuum filtered using a 0.2µm Isopore membrane filter (Millipore Corporation, Herts, UK). The filter is removed, placed onto a glass slide with SlowFade (Molecular Probes, Eugan, OR, USA) and examined under a fluorescent microscope (Nicon Eclipse, E400). The DAPI stained cells are examined under UV light and hybridised cells viewed using a DM510 filter.

Faecal sample are incubated with 1% (w/v) of each substrate. Samples are taken after 24 hours. For each slide at least 15 different fields of view are counted. Microbial counts are presented as $\log_{10}$cells/ml.

PI is calculated with the following equation:

$PI = \Delta B + \Delta L + \Delta E - \Delta Ba - \Delta Cl - \Delta Co - \Delta SRB$

wherein Δ = amount of bacteria in presence of the tested fiber minus the amount of bacteria in absence of the tested fiber; B = *Bifidobacterium* spp; L = lactobacilli; E = *Eubacterium* rectale; Ba = *Bacteroides* spp; Cl = *Clostridium* coccoides and *Clostridium* histolyticum; Co = *Escherichia* coli and SRB = *Desulfovibrio* spp.

Results:

Bacteria	Sucrose	FOS	Δ FOS	tGOS	Δ tGOS	Benefiber®	Δ Benefiber®
Bifidobacteria	8.0	8.9	**0.9**	8.7	**0.7**	8.4	**0.4**
Bacteroides	8.1	8.2	**0.1**	7.8	**-0.3**	9.0	**0.9**
Lactobacilli	6.7	7.0	**0.3**	7.0	**0.3**	6.8	**0.1**
Clostridia	7.6	7.3	**- 0.3**	6.9	**- 0.7**	6.8	**-0.8**
E.Coli	7.7	7.8	**0.1**	6.9	**-0.8**	7.5	**-0.2**
Eubacteria	7.7	8.0	**0.3**	7.7	**0**	8.2	**0.5**
Desulfovibrio	6.7	6.9	**0.2**	7.2	**0.5**	6.9	**0.2**

PI			1.4		2.3		0.9

tGOS shows the highest growth rate of *Bifidobacterium* spp. at the expense of *Bacteroides* spp. and *Eubacterium rectale/Clostridium coccoides* group, as well as the smallest increase in *E. coli.* FOS also results in a high growth rate of bifidobacteria, however, high growth rates of *Clostridium histolyticum* group and *E. coli* are also observed. Benefiber® results in a high growth rate of *Bacteroides* spp. and *E.coli* with a positive effect on bifidobacteria when compared to sucrose.

2.2 Analysis of SCFA

The production of succinic, lactic, formic, acetic, propionic, isobutyric, butyric, isovaleric and valeric acids in the fermentations is quantified. Samples are centrifuged at 1500 x g for 15 minutes and the resultant supernatant used for injection. A Model 1050 UV HPLC (Hewlett Packard), with an integrated oven compartment (50°C) and data system is used in combination with a differential refractometer (Knauer). Sample injection is performed using an autosampler and is of 20µl volume. The column is a pre-packed Aminex HPX-87-H strong cation-exchange resin column (150 x 7.8 mm I.D.), fitted with an ion exclusion micro-guard refill cartridge (Bio-Rad Labs., USA). The eluent used is 0.005 M sulphuric acid. Faecal samples are incubated with135ml basal nutrient medium containing 1% (w/v) sucrose, PHGG, FOS or tGOS in batch culture systems at 37° C. Samples are taken every 2 hours up to 10 hours and then at 15 and 24 hours. Data are not shown.

Results: In the case of sucrose, FOS and tGOS SCFA production is either diminished or constant after 10 hours, whereas Benefiber results in SCFA production up to 24 hours. SCFA profiles are very different between different substrates. Sucrose fermentation results in the production of acetate and very little or no other acids. Acetic and lactic acids are produced with both FOS and tGOS fermentation. FOS fermentation shows very little or no propionate and butyrate production. The main product of Benefiber fermentation is acetic acid followed by propionic and butyric acids. There is very little or no lactate production with Benefiber fermentations.

2.3. Measurement of total sugar by total carbohydrate assay.

A phenol-sulphuric acid assay is used for the determination of total carbohydrate content as expressed in glucose equivalents. The assay is calibrated with D-glucose standards ranging from 0 to 0.15 $mgml^{-1}$. Measurements of the absorbance at 450nm are taken using the spectrophotometer and plotted against the standards. The total carbohydrate content is thus calculated.

Faecal samples are incubated with 0% (w/v), 0.25% (w/v), 0.5% (w/v) and 1% (w/v) of substrate and 135ml basal nutrient medium in batch culture systems at 37° C. Substrate is a) sucrose, b) PHGG, c) FOS and d) tGOS. Samples are taken every 2 hours up to 10 hours and then at 15 and 24 hours (Figure 1a, 1b, 1c and 1d).

Results: The measure of the concentration of total carbohydrate present in each fermentation vessel shows fast fermentation times for sucrose, FOS and tGOS. In all three cases, the substrates are completely utilised by 8 hours regardless of concentration. Although, a fast substrate breakdown up to 8 hours is observed in the case of PHGG some substrate can still be detected in the fermentation vessels up to 24 hours (Figure 1c), suggesting a slower fermentation.

(3) *In vitro* gut model

The conditions in the colon are replicated in a three stage continuous fermenter (Macfarlane *et al.*, 1998) inoculated with 10% (w/v) faecal homogenate from healthy human volunteers in a growth medium without and with 1% (w/v) testing substrate. The model consists of three vessels, V1, V2 and V3, with respective operating volumes of 270, 300 and 300 ml. Temperature is set at 37°C and together with pH is controlled automatically. Culture pH in the three vessels is maintained at 5.5, 6.2 and 6.8, respectively. Each fermenter is magnetically stirred and kept under anaerobic conditions by continuously sparging with O_2-free N_2 (15ml/min). The growth medium contains the following ingredients: starch 8g/l, mucin 4g/l, casein 3g/l, peptone water 5g/l, tryptone water 5g/l, bile N°3 0.4g/l, yeast, 4.5 g/l, $FeSO_4$ 0.005g/l, NaCl 4.5g/l, KCl 4.5g/l, KH_2PO_4 0.5g/l, $MgSO_4.7H_2O$ 1.25g/l, $CaCl_2.6H_2O$ 0.15g/l, $NaHCO_3$ 1.5g/l, Tween 80 1ml, Hemin 0.05g/l, Cysteine.HCl 0.8g/l. The medium is fed to V1 by a peristaltic pump and V1 sequentially supplies V2 and V3 through a series of tubes. The system is operated at a retention time of about 36 hours. The gut model is left overnight to equilibrate before the medium pump is switched on and is run for 10.5 days before medium containing testing substrate is introduced and it is then left for further 10.5 days. Samples are taken at the beginning and the end of each cycle. The sample volume removed is 5 ml

and this amount is used for SCFA analyses, FISH and total carbohydrate measurement.

3.1. Fluorescence *in situ* hybridisation (FISH)

Faecal sample are incubated with 1% (w/v) of each substrate. Samples are taken after 21 days. For each slide at least 15 different fields of view are counted. Microbial counts are presented as log_{10}cells/ml.

PI is calculated with the following equation:

$PI = \Delta B + \Delta L + \Delta E - \Delta Ba - \Delta Cl - \Delta Co - \Delta SRB$

Wherein Δ = amount of bacteria in presence of the tested fiber minus the amount of bacteria in absence of the tested fiber; B = bifidobacteria; L = lactobacilli; E = Eubacteria; Ba = Bacteroides; Cl = Clostridia; Co = E coli and SRB = Desulfovibrio.

Total PI is calculated by summing PI for each vessel. The base line for these values is gut model medium.

	Gut model medium			FOS			tGOS			PHGG		
Bacteria	V1	V2	V3	V1	V2	V3	V1	V2	V3	V1	V2	V3
Bifidobacteria	8.1	8.0	8.0	8.4	8.3	8.1	8.3	8.3	8.3	8.0	8.0	8.2
Bacteroides	8.0	8.2	8.1	7.7	7.7	7.8	7.5	7.5	7.5	8.0	8.7	8.7
Lactobacilli	7.1	7.0	6.9	7.1	7.1	7.0	7.1	7.1	6.9	7.1	7.1	7.0
Clostridia	6.8	6.8	6.9	6.8	6.8	6.8	7.1	7.1	7.0	7.2	6.9	6.6
E. Coli	6.9	6.9	7.1	7.2	7.0	7.1	7.2	7.1	7.2	7.0	7.2	7.2
Eubacteria	7.7	7.9	8.0	7.5	7.6	7.4	7.5	7.5	7.5	7.8	7.4	8.3
Desulfovibrio	7.2	7.2	7.3	7.0	7.2	7.2	6.7	6.9	6.9	7.6	7.7	7.8

PI	FOS	GOS	PHGG

V1	0.4	1.6	-0.9
V2	0.5	1.0	-1.6
V3	1.0	0	-0.3
Total PI	**1.9**	**2.6**	**-2.8**

Results: From both batch cultures and gut models, GOS seems to have the best effect. GOS and FOS have a similar PI in both batch and gut models.

3.2. Analysis of SCFA

The same protocol is used as for batch fermentation. The SCFA profiles measured for gut models containing Benefiber, FOS and GOS as substrates are presented in Figures 2, 3 and 4, respectively.

Results: In general, lactic acid production is low throughout all vessels and with all substrates assessed. The only increase is observed with the addition of FOS in the first vessel (Figure 3a). Similarly, acetic acid production is increased only in the first vessel in the gut model containing FOS (Figure 3a) and decreased in the first vessel containing Benefiber (Figure 2a), whilst the rest shows no change. The addition of FOS and tGOS into the medium results in no observed effect upon production of propionic acid in any of the vessels, whilst the addition of Benefiber results in increased production of propionic acid in all three vessels. Butyric acid production is increased with the addition of tGOS and Benefiber in all three vessels. The addition of FOS has no effect on the production of butyric acid in any vessel. Generally, the SCFA profiles demonstrates that both tGOS and Benefiber in particular, have an effect in all three vessels whilst FOS has the most pronounced effect on the first vessel and very little effect upon the second and third vessels.

Table 1. Oligonucleotide probes used for the characterisation of gut microflora using FISH

Probe	Sequence	Target genus	Temperature
Bac 303	5'-CCAATGTGGGGGACCTT-3'	*Bacteroides* spp.	45°C
Bif 164	5'-CATCCGGCATTACCACCC-3'	*Bifidobacterium* spp.	50°C
Erec 482	5'-CGGUACCUGACUAAGAAGC-3'	*Clostridium coccoides-Eubacterium rectale* group	50°C
Chis 150	5'-AAAGGAAGAUUAAUACCGCAUA-3'	*Clostridium histolyticum* group	50°C
Ec 1531	5'-CACCGTAGTGCCTCGTCATCA-3'	*E.coli*	37°C
Lab 158	5'-GGTATTAGCA(T/C)CTGTTTCCA-3'	*Lactobacillus/Enterococcus* spp.	45°C
Srb 687	5' – TACGGATTTCACTCCT-3'	*Desulfovibrio spp.*	48°C

Claims

1. A method for evaluating the prebiotic capability of a fiber, which comprises a subtractive culture method for evaluating or quantifying the stimulation by the tested fiber on the growth of a faecal bacterial population and/or on the modification of the faecal bacterial population.

2. The method according to claim 1 wherein the evaluation or quantification of the stimulation by the tested fiber of the growth of a faecal bacterial population comprises
(a) incubating a faecal bacterial culture in parallel in the presence and in the absence of the tested fiber,
(b) after a certain incubation period, for instance 24 hours, determining the amount of the faecal bacteria in the culture in the presence and in the absence of the tested fiber,
(c) subtracting the amount of the faecal bacteria in the absence of the tested fiber from the amount of the faecal bacteria in the presence of the tested fiber,
(d) evaluating the prebiotic capability of the tested fiber as a function of the value as determined in step (c).

3. A method for evaluating the prebiotic capability of a fiber or for identifying a prebiotic substance, which comprises
(a) incubating the same faecal bacterial culture in the presence and in the absence of the tested fiber,
(b) determining the amount of the beneficial faecal bacteria in the presence and in the absence of the tested fiber,
(c) subtracting the amount of the beneficial faecal bacteria in the absence of the tested fiber from the amount of the beneficial faecal bacteria in the presence of the tested fiber,
(d) determining the amount of the non beneficial faecal bacteria in presence and in the absence of the tested fiber,.
(e) subtracting the amount of the non beneficial faecal bacteria in the absence of the tested fiber from the amount of the non beneficial faecal bacteria in the presence of the tested fiber,
(f) subtracting the value as determined in step (e) from the value as determined in step (c) to produce a Prebiotic Index,

(g) evaluating the prebiotic capability of the tested fiber as a function of the Prebiotic Index.

4. The method according to claim 3 wherein the beneficial faecal bacteria is at least one of bifidobacteria, lactobacilli and Eubacteria.

5. The method according to claim 3 or 4 wherein the non beneficial faecal bacteria is at least one of bacteroides, clostridia, coliforms, and Sulfate Reducing bacteria.

6. The method according to claim 3 wherein the identification and quantification of the Prebiotic Index (PI) is defined by the following equation:
$PI = \Delta B + \Delta L + \Delta E - \Delta Ba - \Delta Cl - \Delta Co - \Delta SRB$,
wherein Δ = amount of bacteria in presence of the tested fiber minus the amount of bacteria in absence of the tested fiber; B = bifidobacteria, L = lactobacilli, E = Eubacteria, Ba = bacteroides, Cl = clostridia, Co = coliforms, and SRB = Sulfate Reducing bacteria.

7. The method according to any preceding claim which comprises a step of quantifying the fermentation end products.

8. The method according to any preceding claim which comprises a step of quantifying the rate of fiber breakdown.

9. The method according to any preceding claim which comprises a step of determining the fermentation time, e.g. the relationship between the growth of the faecal bacterial population and the tested fiber concentration.

10. The method according to claim 3 wherein the evaluation of the prebiotic capability of the tested fiber is a function of the Measure of the Prebiotic Effect (MPE), and the MPE is defined using the following equation:

$$\frac{1}{2}\sqrt{x^2y^2 + x^2z^2 + y^2z^2} = MPE$$

wherein x = modification in the faecal bacterial population; y = quantification of the fermentation end products; z = rate of fiber breakdown.

11. A method according to any preceding claim wherein the faecal bacteria population is analyzed by fluorescence *in situ* hybridization.

12. A method according to any preceding claim wherein the tested fiber is FOS and/or GOS.

13. A method according to any preceding claim which comprises a step of comparing the prebiotic capability of the tested fiber to the effect of a known prebiotic tested in the same conditions.

14. A method for designing a nutritional or pharmaceutical composition containing a prebiotic, which method comprises
(a) evaluation and optionally quantification of the prebiotic capability of a fiber with the method of any one of claims 1 to 12
(b) selecting at least one fiber having a good prebiotic capability, e.g. comparable to the prebiotic activity of a known prebiotic, and
(c) formulation of a nutritional or pharmaceutical composition comprising the prebiotic selected in step (b) and a nutritionally or pharmaceutically acceptable carrier.

15. A method for providing a nutritional or pharmaceutical composition containing a prebiotic to an individual in need thereof, which method comprises
(a) evaluation and optionally quantification of the prebiotic capability of a fiber with the method of any one of claims 1 to 12
(b) selecting at least one fiber having a good prebiotic capability, e.g. comparable to the prebiotic activity of a known prebiotic, and
(c) formulation of a nutritional or pharmaceutical composition comprising the prebiotic selected in step (b) and a nutritionally or pharmaceutically acceptable carrier, and
(d) providing the nutritional or pharmaceutical composition obtained in step (c) to an individual in need thereof.

16. A system for designing a nutritional or pharmaceutical composition containing a prebiotic and delivering said nutritional or pharmaceutical composition to an individual in need thereof, which system comprises

(a) means for quantifying the effect of a fiber on faecal bacteria and identification of a prebiotic,
(b) means for formulating of a nutritional or pharmaceutical composition comprising the prebiotic identified in step (a) and a nutritionally or pharmaceutically acceptable carrier, and
(c) means for providing the nutritional or pharmaceutical composition obtained in step (b) to an individual in need thereof.

17. A nutritional or pharmaceutical composition obtained by the method of claim 15.

18. Use of GOS and/or FOS to stimulate the growth of bifidobacteria and/or lactobacilli, and/or to inhibit the growth of at least one of Bacteroides, Eubacteria, Clostridia, Coliforms, Sulfate reducing Bacteria.

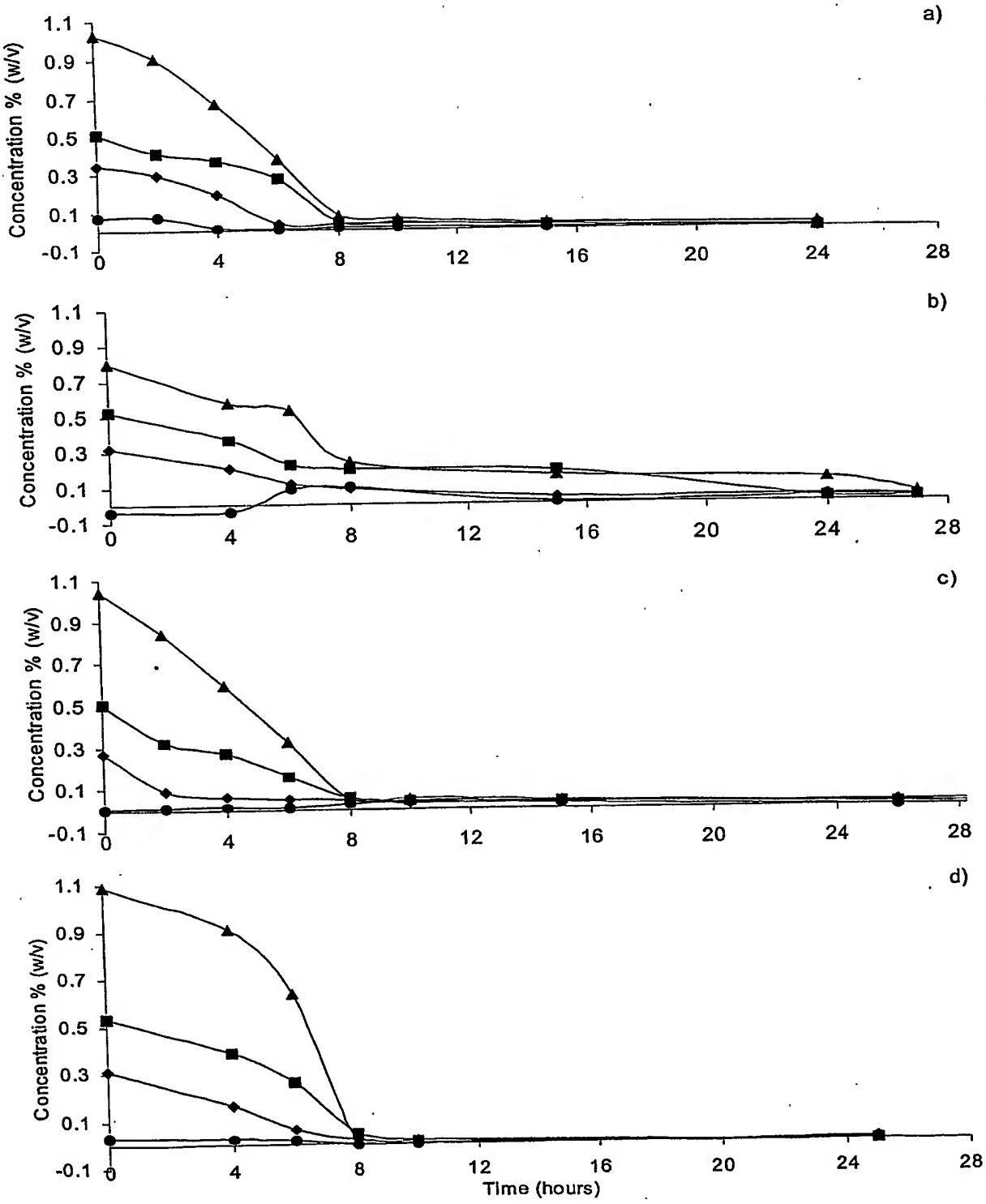
19. Use according to claim 18 wherein the bifidobacteria are *Bifidobacterium* spp., the Bacteroides are *Bacteroides* spp., the Eubacteria are *Eubacterium rectale*, the Clostridia are *Clostridium histolyticum or Clostridium coccoides*, the Coliforms are *E. coli* and the Sulfate reducing Bacteria are *desulfibrio.*

20. Use of a composition comprising GOS and/or FOS for the manufacture of a nutritional or pharmaceutical composition for stimulating the growth of endogenous bifidobacteria and/or lactobacilli, and/or for inhibiting the growth of at least one of endogenous Bacteroides, Eubacteria, Clostridia, Coliforms and Sulfate reducing Bacteria.

Abstract

The present invention concerns a method for providing a nutritional and pharmaceutical composition comprising a prebiotic to an individual in need thereof.

Figure 1/4:


a)
b)
c)
d)
Concentration % (w/v)
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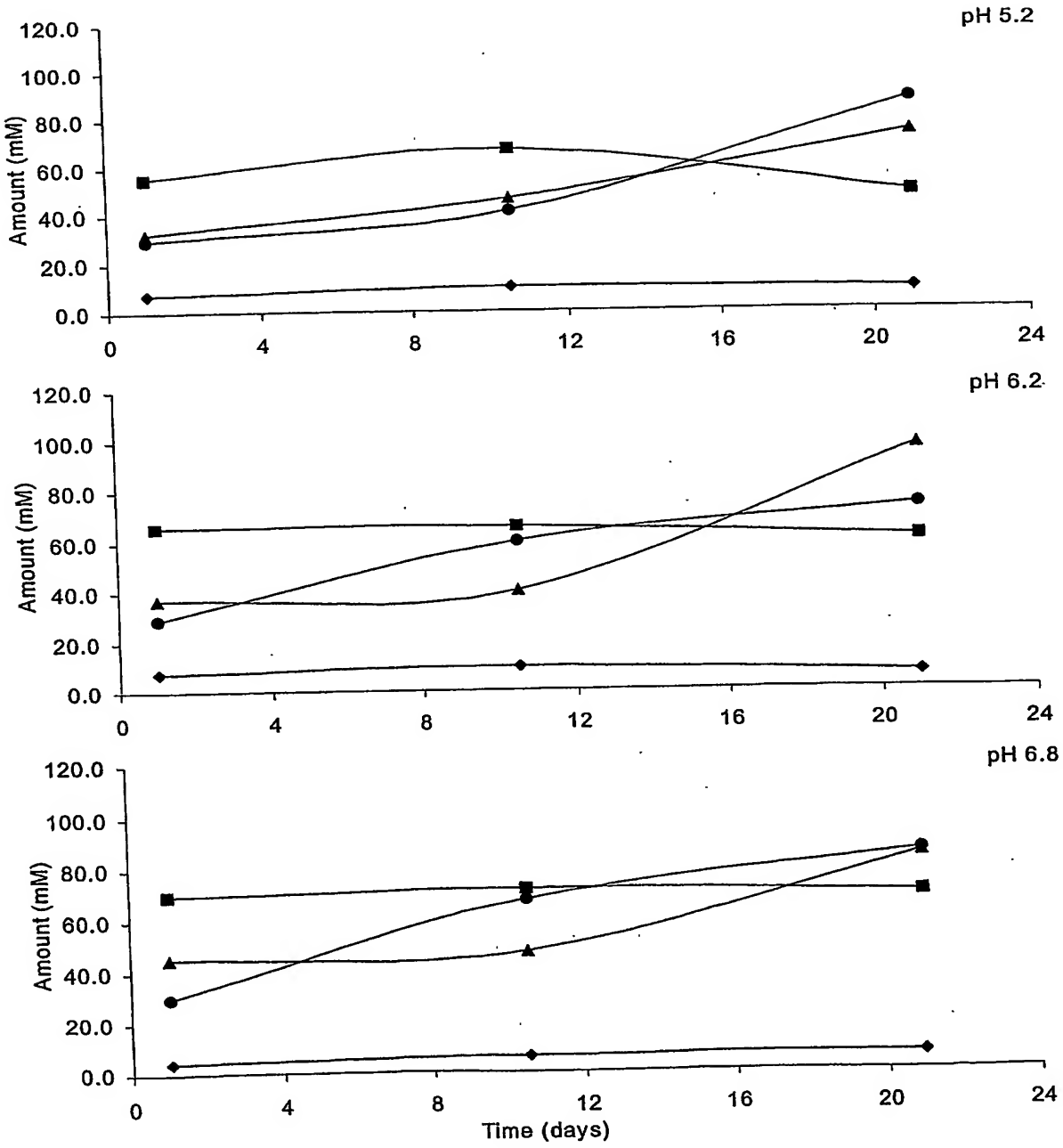
0.9
0.7
0.5
0.3
0.1
-0.1
0
4
8
12
16
20
24
28
Time (hours)

Figure 2/4: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20.0
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Amount (mM)
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8
12
16
20
24
pH 6.2
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Amount (mM)
0
4
8
12
16
20
24
pH 6.8
120.0
100.0
80.0
60.0
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Amount (mM)
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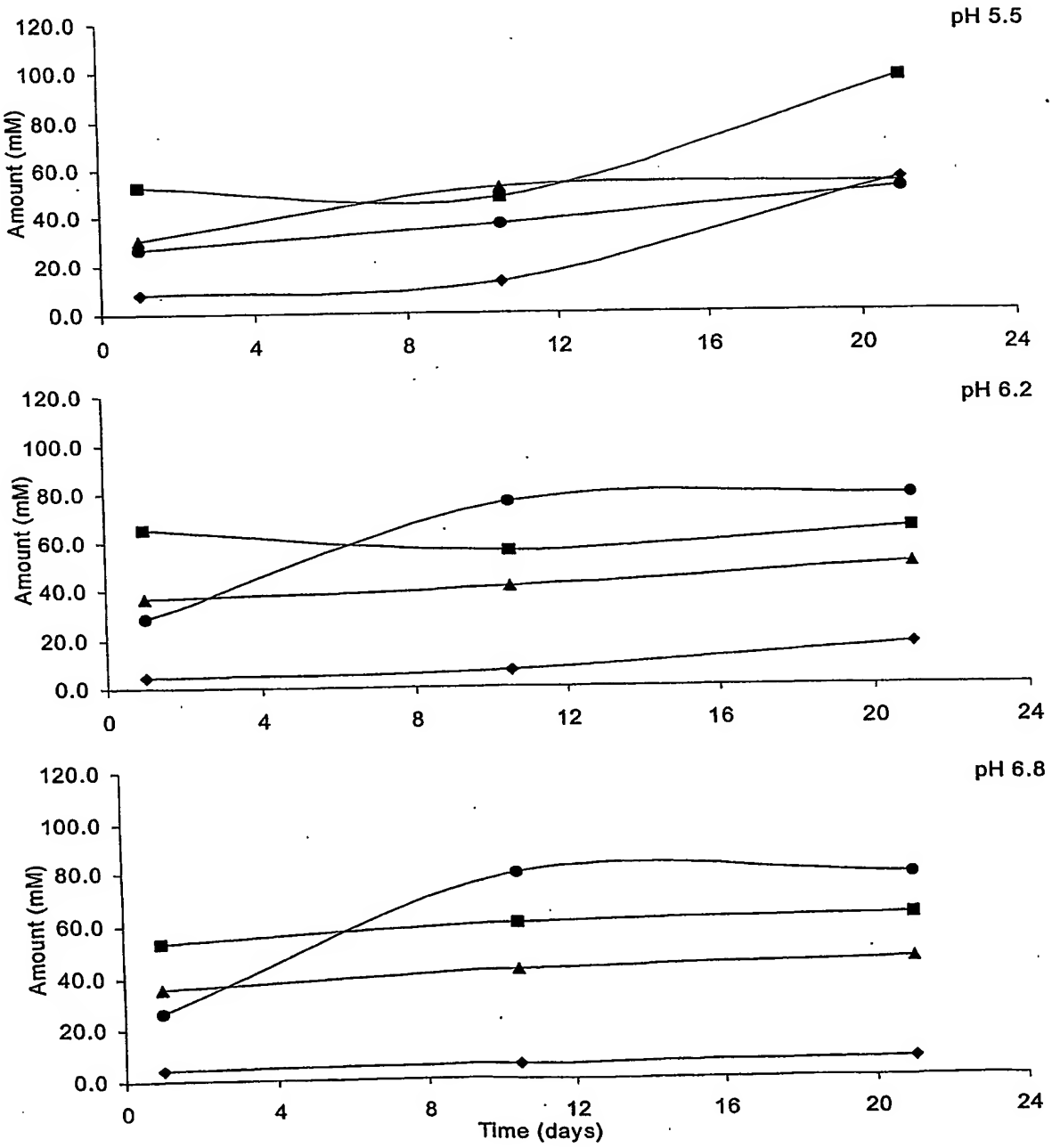
4
8
12
16
20
24
Time (days)

Figure 3/4: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16
20
24
pH 6.2
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Amount (mM)
0
4
8
12
16
20
24
pH 6.8
120.0
100.0
80.0
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20.0
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Amount (mM)
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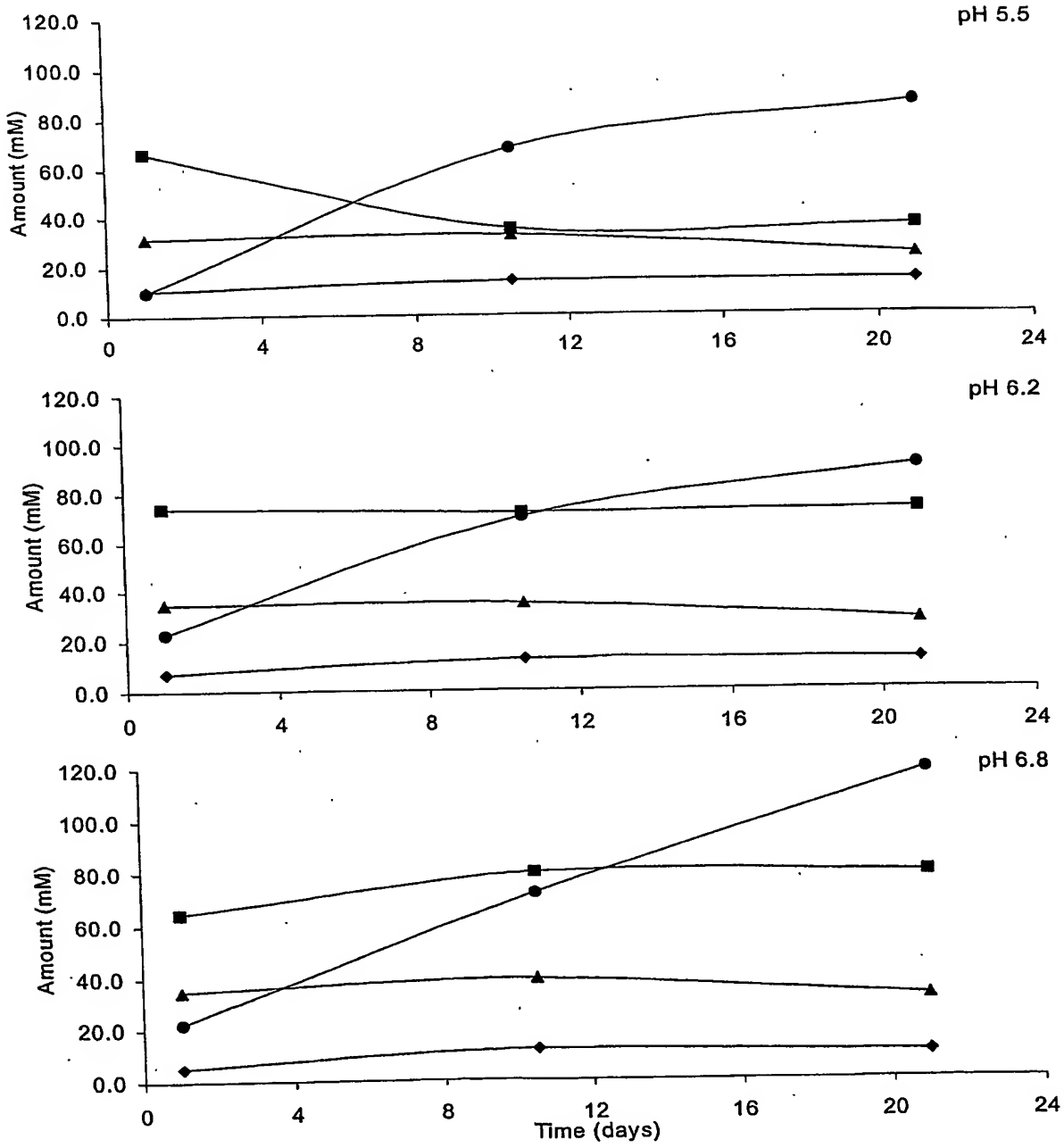
12
16
20
24
Time (days)

Figure 4/4: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60.0
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4
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16
20
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pH 6.2
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Amount (mM)
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16
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pH 6.8
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Amount (mM)
0
4
8
12
16
20
24
Time (days)